From Department of Oncology-Pathology
Cancer Center Karolinska
Karolinska Institutet, Stockholm, Sweden

UNDERSTANDING P53 STRUCTURE AND TARGETING MUTANT P53 FOR IMPROVED CANCER THERAPY

Qiang Zhang

Karolinska Institutet

Stockholm 2018

Published by Karolinska Institutet.
Printed by Eprint AB 2018

ISBN **978-91-7831-084-5**

Understanding p53 structure and targeting mutant p53 for improved cancer therapy

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Lecture Hall, Radiumhemmet, Karolinska Hospital, Solna

Friday June 15th, 9:30 am

By

Qiang Zhang

Principal Supervisor:
Professor Klas G. Wiman
Karolinska Institutet
Department of Oncology-Pathology

Co-supervisor:
Docent Vladimir J.N. Bykov
Karolinska Institutet
Department of Oncology-Pathology

Opponent:
Professor Thanos Halazonetis
University of Geneva
Department of Molecular Biology

Examination Board:
Professor Martin Bergö
Karolinska Institutet
Department of Biosciences and Nutrition

Professor Stig Linder
Linköping University and Karolinska Institutet
Department of Medical and Health Sciences
Department of Oncology-Pathology, KI

Docent Per Jemth
Uppsala University
Department of Medical Biochemistry and Microbiology

To My family

献给我的家人

ABSTRACT

The p53 gene family consists of p53, p63 and p73. The three proteins share a high degree of structural similarity in their DNA-binding domains but have rather different functions. The tumor suppressor p53 acts as the guardian of the genome and is activated in response to cellular stress. p53 is a transcription factor that activates downstream target genes to induce cell cycle arrest, senescence or apoptosis. However, the TP53 gene is inactivated by missense mutation in about half of human tumors. Therefore, reactivation of mutant p53 is an attractive strategy for novel cancer treatment. The mutant p53-reactivating compound PRIMA-1, identified in a cellular screen of the NCI Diversity set, suppresses tumor cell growth in a mutant p53-dependent manner. The methylated analog PRIMA-1Met, now named APR-246, is even more potent. APR-246 has been successfully tested in a phase I/IIa clinical trial. A phase II clinical trial in ovarian cancer is ongoing. Both PRIMA-1 and APR-246 are prodrugs that are converted to the active compound methylene quinuclidinone (MQ), a Michael acceptor that binds covalently to cysteines in p53's DNA-binding domain.

In paper I, we tested the effect of APR-246 on primary adult skin keratinocytes with p63 mutations from patients with the EEC developmental syndrome. We showed that APR-246 can partially rescue morphological defects of the mutant p63-carrying EEC keratinocytes, and expression of differentiation and stratification markers. Furthermore, we found that APR-246 restored the expression of p63 target genes. Our findings demonstrate that APR-246 can also target mutant forms of p63.

In paper II, we found that the Michael acceptor activity of MQ is critical for MQ binding, thermostabilization and refolding of mutant p53. We identified Cys277 as a prime binding site for MQ in p53. Cysteine to alanine substitution at this position abolished both MQ binding and thermostabilization. Moreover, we found that both Cys124 and Cys277 are required for APR-246 mediated mutant p53 reactivation in His175 mutant p53-carrying tumor cells.

In paper III, we selected potential thiol-reactive compounds including Michael acceptors, primary alcohols, imines and aldehydes, with top p53 selectivity based on datamining of the NCI database. Multivariable analysis identified different functional groups associated with various features of mutant p53 reactivation. Michael acceptors are more prone to high toxicity and thiol reactivity. Alcohols and imines are more associated with p53 refolding. Aldehydes are more likely to stabilize p53. These results may facilitate the design of novel mutant p53-targeting compounds.

Paper IV describes a p53-like gene in the hydrothermal annelid *Alvinella pompejana*. Sequence alignment and structure modeling indicated that this p53 homolog is more similar to p63 and p73. Consistent with this finding, the DNA-binding domain of *Alvinella* has high thermostability. We identified repacking features in the hydrophobic core of *Alvinella* p53 that are associated with its high thermostability. Understanding structural features of p53

family proteins that govern stability may provide insights for development of mutant p53-reactivating drugs.

LIST OF SCIENTIFIC PAPERS

I. Shen, J., van den Bogaard, E.H., Kouwenhoven, E.N., Bykov, V.J., Rinne, T., **Zhang. Q.,** Tjabringa, G.S., Gilissen, C., van Heeringen, S.J., Schalkwijk, J., van. Bokhoven. H., Wiman. K. G., Zhou. H.
APR-246/PRIMA-1(MET) rescues epidermal differentiation in skin keratinocytes derived from EEC syndrome patients with p63 mutations.
Proc Natl Acad Sci U S A **110**, 2157-2162 (2013).

II. **Zhang. Q.,** Bykov V., Wiman. K. G., Zawacka-Pankau. J.
APR-246 reactivates mutant p53 by targeting cysteines 124 and 277.
Cell Death Dis **9**, 439 (2018)

III. **Zhang. Q.,** Bergman. J., Wiman. K. G., Bykov V.
Role of thiol reactivity for targeting mutant p53. *Manuscript.*

IV. **Zhang. Q.,** Baron. B., Wiman. K. G., Joerger, A.C., Soussi. T.
The DNA-binding domain of a p53 homolog from the hydrothermal annelid *Alvinella pompejana*: protein stability and evolutionary history of the p53 family. *Manuscript*.

CONTENTS

1 Introduction 13
1.1 Cancer 13
1.2 Cancer therapy 13
1.3 p53 14
1.3.1 Structure of p53 14
1.3.2 Regulation of p53 16
1.3.3 p53 function 16
1.3.4 p53 family member p63 and p73 18
1.3.5 p53 inactivation and cancer 20
1.4 Rescue of wild-type p53 function for cancer therapy 21
1.4.1 Activation of wild-type p53 21
1.4.2 Reactivation of mutant p53 21
2 Aims of the thesis 25
3 Results and discussions 27
3.1 Paper I 27
3.2 Paper II 29
3.3 Paper III 30
3.4 Paper IV 31
4 Conclusions and future perspectives 33
5 Acknowledgements 35
6 References 39

LIST OF ABBREVIATIONS

ADH7	Alcohol dehydrogenase 7
AEC	Ankyloblepharon-ectodermal defects-cleft lip/plate
BS	Binding sites
Bax	Bcl2-associated X protein
CD	Circular dichroism
CDK	Cyclin-dependent kinase
DBD	DNA binding domain
DMF	Dimethyl fumarate
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
DSF	Differential scanning fluorimetry
EEC	Ectrodactyly, ectodermal dysplasia, and cleft lip/palate
EST	Expressed sequence tag
GSH	Glutathione
GPX2	Glutathione peroxidase 2
K1	Keratin 1
MDM2	Mouse double minute 2 human homolog
MQ	Methylene quinuclidinone
MIRA-1	Mutant p53-depedent induction of rapid apoptosis
MTD	Maximum tolerated dose
NAC	N-acetylcysteine
NCI	National Cancer Institute
PRIMA-1	p53 reactivation and induction of massive apoptosis
ROS	Reactive oxygen species
SAM	Sterile alpha motif
STIMA-1	SH targeting compound that induces massive apoptosis

SV40	Simian virus 40
TAD	Transactivation domain
TMG	Transglutaminase
TrxR1	Thioredoxin reductase
UTR	Untranslated region

1 INTRODUCTION

1.1 CANCER

Cancer is one of the most lethal diseases that threaten the health of human beings. There were 14 million new cases of cancer in the world in 2012 and 8.8 million people worldwide died from cancer in 2015 according to the World Health Organization (WHO) (http://www.who.int/Topics/cancer/en). Unlike normal cells that differentiate to distinct cell types with specific functions and stop growing and die as part of normal physiological processes or when damaged, cancer cells grow rapidly before they have had a chance to mature, they do not die and essentially become immortal. A tumor can potentially spread to other part of the body.

There are more than 100 different types of cancer. Types of cancer are usually named for the organs or tissues where the cancers located, such as brain cancer, lung cancer, etc. or the type of cells formed them for example, Squamous cell carcinoma, glioma, etc. All types of cancer shares the same features or Hallmarks as defined by Hanahan and Weinberg (Hanahan and Weinberg, 2011). (1) Keep dividing signals on; (2) evading from growth suppression; (3) avoiding immune destruction; (4) have unlimited replication potential; (5) inducing tumor-associated inflammatory response that enhances tumorigenesis and progression; (6) invasion of cells to surrounding tissues and metastasis; (7) form new blood vessels around the tumor to get nutrition; (8) genetically instable; (9) resistant to cell death; (10) altered energy metabolism.

Cancer is a genetic disease that is caused by the alteration of genes. The changes of genes are mainly caused by environmental factors, such as, radiation, chemicals, diet and obesity, tobacco, infections, etc. The remaining is due to inherited genetics. However, not all the genetic changes cause cancer; some of them have nothing to do with cancer or may be just the results of cancer. The changes of genes that contribute to carcinogenesis are called “driver” of cancer. The “driver” genes mainly belong to three types of genes, DNA repair genes, proto-oncogenes and tumor suppressor genes. The alterations of DNA repair genes lead to additional mutations and promotion of carcinogenesis. Proto-oncogenes are necessary in normal cell growth but can convert to oncogenes when they are altered. Tumor suppressor genes are guardians in the body to control cell growth but mutations in those genes may result in loss of the supervision functions and even new functions to stimulate tumor growth.

1.2 CANCER THERAPY

Surgery, radiotherapy and chemotherapy are most common treatments for cancer and they can be used either alone or combined with each other. Surgery is the most effective method for isolated, primary solid cancer. In certain cases, patients can be cured by removing the tumors and the surrounding tissues as well as associated regional lymphatics. However, surgery is not optimal when tumors are hardly reached or metastatic. Radiotherapy is also widely used in cancer treatment. It uses ionizing radiation to damage the DNA of cancerous

tissues to kill them. Radiotherapy can be used as curative treatment for many types of cancer for example early stage head and neck cancer as well as prostate cancer. Traditional chemotherapy targets fast dividing cells and disrupts cell mitosis. Most of chemotherapeutic drugs kill cancer cells by damaging DNA and inhibiting cellular machinery involved in cell division. However, some normal cells also proliferate rapidly such as hair follicles and bone marrow thus rendering them sensitive to chemotherapeutic drugs, and subsequently leading to side effects. In order to kill only cancer cells but not normal cells, targeted cancer therapy has arisen. Instead of inhibiting all types of rapidly dividing cells, targeted cancer therapies suppress cancer cells growth by interfering with specific molecular targets involved in tumorigenesis. Currently, there are mainly two types of targeted cancer therapies: small molecules and monoclonal antibodies. Small molecules act by targeting specific enzymes such as tyrosine-kinase inhibitors, blocking growth factors and activating tumor suppressors. Monoclonal antibodies act by stimulating patients' immune system to attack cancer cells.

1.3 p53

In 1979, p53 was discovered by several independent groups studying simian virus 40 (SV40). However, p53 was mistakenly defined as an oncogene in the beginning. This is because the p53 was obtained from the cancer cells was mutant. However, around 1988-89 results from several groups showed that wild-type p53 is a tumor suppressor, milestone in this fantastic field. The exact molecular mass of p53 is 43.7 kDa. Why its name is p53? The reason is it migrates as 53 kDa band on the SDS–PAGE gel due to the high number of proline residues in the protein.

1.3.1 Structure of p53

In humans, the TP53 gene is located on chromosome 17p13.1. It contains 11 exons. The translation starts from the second exon and generates a 393 amino acid protein. The structure of p53 protein is characterized by an N-terminal transactivation domain (TAD), a DNA binding domain (DBD) and a C-terminal tetramerazation domain (Figure. 1).

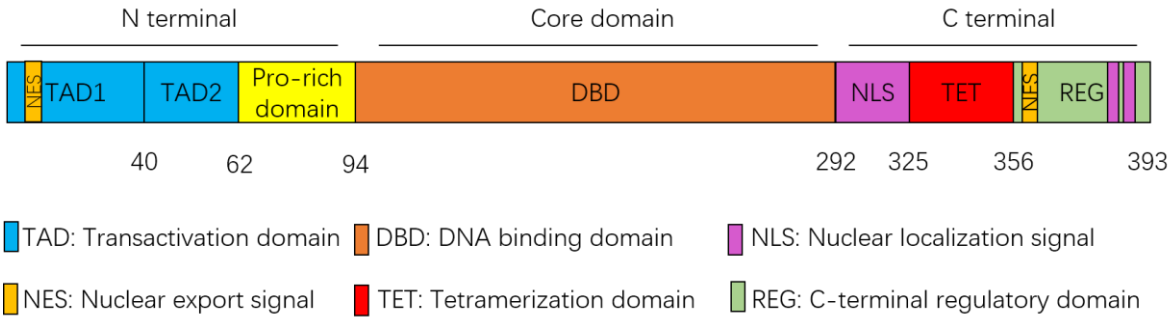

Figure 1. Schematic representation of the p53 protein.

The transactivation domain of p53 consists of two subdomains TAD1 and TAD2. Both subdomains contain Φ-X-X-Φ-Φ motifs (Φ indicates a bulky hydrophobic residue and X is

any other residue) but display independent transcriptional activities. TAD1 specifically binds to MDM2 (Kussie et al., 1996), which negatively regulates p53 stability. TAD2 interacts with the replication protein A (RPA70) that blocks p53 binding to specific DNA (Bochkareva et al., 2005). The activity of TAD is regulated by post-translational modification, mainly phosphorylation. Phosphorylation of Ser15, Thr18 and Ser20 reduce p53-MDM2 complex formation (Bode and Dong, 2004). Phosphorylation of ser46 by HIPK-2 or DYRK-2 affects induction of p53 proapoptotic target gene such as p53-regulated Apoptosis-Inducing Protein 1 (p53AIP1) (Oda et al., 2000).

Between the transactivation domain and the DNA binding domain there is a proline-rich domain that contains five repeats of PXXP sequence. Lack of this region results in deficiency in anti-neoplastic agents induced cell death (Baptiste et al., 2002).

The DNA binding domain of p53 is located from residues 94 to 292. Crystal structure of p53 shows that the basic structure of p53 DNA binding domain is an immunoglobulin like sandwich formed by two anti-parallel β-sheets that contains four and five small β-strands respectively (Cho et al., 1994). Loop2 occurs between S4 and S5, and Loop3 occurs between S8 and S9 are stabilized by a loop-sheet-helix motif (loop L1, beta-strands S2 and S2', four C terminal residues of the extended beta-strand S10, and the helix H2). The motif and two loops create the DNA binding surface of p53. Between L2 and L3 there is a zinc atom coordinated by Cys176, His179, Cys238 and Cys242, which is critical for maintaining the structure of p53 protein. The binding of p53 to DNA can be described in three principal parts: Lys120, Cys277 and Arg280 bind to the major groove of DNA; Arg248 bins to minor groove of DNA; Ser241, Ala276, Arg273 and Arg283 bind to the backbone of DNA. There are 10 cysteines (Figure. 2) in the DNA binding domain of p53 with different solvent accessibilities. Cys277 and Cys182 are the most accessible cysteines followed by Cys229, Cys242 and Cys124. Cys135, Cys141 and Cys275 are buried in the hydrophobic core and have no solvent accessibility (Scotcher et al., 2011).

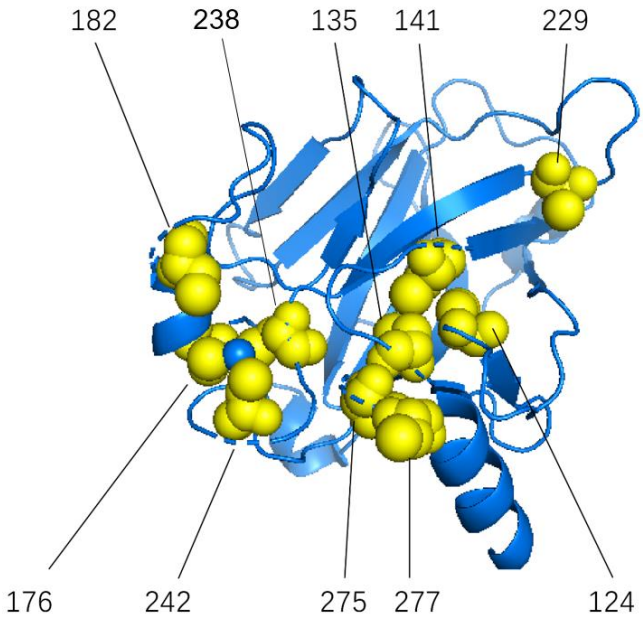

Figure 2. Cysteines（Yellow）in p53 the core domain. (PDB ID 2OCJ; figures generated using PyMOL).

Three nuclear localization signals (NSLs) (Shaulsky et al., 1990) were identified in p53 protein. They together with two nuclear export signals (NESs) (Stommel et al., 1999) regulate p53 protein nuclear localization. The tetramerization domain is essential for p53 forming tetramers that plays important roles for DNA binding, protein-protein interaction and p53 degragation (Chene, 2001). The basic C-terminal regulation domain binds to DNA nonspecifically but affects the affinity of p53 DBD to its specific binding sequences (Hamard et al., 2012).

1.3.2 Regulation of p53

In normal unstressed cells, wild-type p53 maintains in a very low level. The p53 protein is extremely unstable with a 5 -20 minutes half-life (Giaccia and Kastan, 1998). This is because a p53 negative regulator murine double minute 2 (MDM2) that binds to the TAD of p53 and leads to p53 degradation. Residues Phe19, Trp23, and Leu26 in p53 are critical for the binding (Bottger et al., 1997). Phosphorylation of The18 in p53 TAD abrogates p53-MDM2 interaction (Sakaguchi et al., 2000; Schon et al., 2002). MDM2 is an E3 ubiquitin ligase that induces p53 mono- and polyubiquitination. In unstressed condition, low level of MDM2 induces p53 monoubiquitination and nuclear export. In stressed condition, p53 upregulates MDM2 and overexpressed MDM2 forms complexes with p300/CBP and subsequently mediate p53 polyubiqutination (Grossman et al., 1998). Then polyubiquitinated p53 is degraded by proteasome. In addition, it has been shown Lysine residues in p53, mainly in the C-termini, are important for MDM2 mediated p53 degradation (Rodriguez et al., 2000). MDMX, a homolog of MDM2, regulates p53 by binding to p53 TAD and inhibits p53 transactivation activity. Mouse studies have shown that MDM2/MDMX knockout is embryonically lethal but can be rescued by absence of p53 (Jones et al., 1995; Parant et al., 2001). ARF can stabilize p53 by binding to the RING finger domain of MDM2 to inhibit the E3 activity of MDM2 (Honda and Yasuda, 1999). Ribosomal protein L1 binds to and inhibits MDM2, leads to p53 stabilization and activation (Lohrum et al., 2003).

In addition to protein level, p53 is also regulated at mRNA level. Wrap53, an antisense regulator of p53, overlaps the first exon of p53. Binding of wrap53α transcript to p53 mRNA 5’ UTR is responsible for stabilization of p53 mRNA (Mahmoudi et al., 2009). Wig-1 (Vilborg et al., 2009) and human antigen R (HuR) (Zou et al., 2006) stabilize p53 mRNA by binding to the AU-rich elements (ARE) in the 3’ UTR.

1.3.3 p53 function

p53 is activated in response to various stresses such as DNA damage, hypoxia, oncogenic stress (Halazonetis et al., 2008). Depending on types of and extent of stress stimuli and the extent of DNA damage, p53 decides the fate of stressed cells by inducing target genes involved in corresponding pathways, including cell cycle arrest, apoptosis and senescence.

When the stress is minor and the DNA damage is repairable, p53 transcriptionally activates target genes involved in cell cycle arrest such as p21 and 14-3-3σ. p21 inhibits CDK2/cyclin E induced phosphorylation of pRb. Once pRb is phosphorylated, E2F will be released from the pRb-E2F complex and will activate genes involved in progression to S phase (Delavaine and La Thangue, 1999). p53 induces G2/M arrest by repressing Cdc25C, a phosphatase activating M-phase specific kinase cdc2/cyclin B (Clair and Manfredi, 2006). Additionally, p53 transcriptionally activates 14-3-3σ, which prevents the proper nuclear location of cdc2-cyclin B1 complex (Hermeking et al., 1997).

p53 also induces senescence, a permanent cell cycle arrest. When cells become senescent, they lose the capacity to proliferate and have a larger and flattened morphology. Many makers are used for identifying senescent cells for example senescence-associated β-galactosidase (SA-βgal), senescence-associated DNA-damage foci (SDFs), p16, etc (Campisi and di Fagagna, 2007). Senescence can be induced by many factors, such as dysfunctional telomeres (Victorelli and Passos, 2017), persistent oncogenic signaling (Di Micco et al., 2006) and reactive oxygen species (ROS) triggered intracellular oxidative damage (Weyemi et al., 2012). Studies have shown inactivation of p53 suppresses senescence (Beausejour et al., 2003; Kumar et al., 2011).

In DNA repair pathway, p53 transcriptional activates DNA damage binding protein 2 (DDB2), which strongly binds to damaged DNA and facilitates the nucleotide excision repair (NER) pathway (Zou et al., 2016). p53 binds to MSH2 involved in DNA-mismatch repair (Scherer et al., 1996).

p53 has been shown to induce autophagy by transactivation of the damage-regulated autophagy-modulator (DRAM) (Crighton et al., 2006). Paradoxically, p53 also been shown to inhibit autophagy (Tasdemir et al., 2008). p14ARF is a potent inducer of autophagy repressed by p53. Inactivation of 53 leads to high expression of p14ARF thereby inducing autophagy (Abida and Gu, 2008).

When the damage is severe and can not be repaired, p53 will induce apoptosis, a programed cell death process, to eliminate damaged cells. It is a critical way for suppressing tumorigenesis. p53 regulates apoptosis by both extrinsic and intrinsic pathways. The extrinsic pathway recruits death receptors to form Death-Inducing-Signaling-Complex (DISC), which leads to caspase cascade activation and to apoptosis. Fas/CD95 is the most common death receptor and a key component for extrinsic pathway (Nagata and Golstein, 1995). Upon p53 transactivation Fas is activated by binding to its ligand FasL and activates caspase-8. The intrinsic pathway governs the release of cytochrome-C from the mitochondria by Bcl-2 family members such as, Bax, PUMA and Noxa. Once be transactivated by p53, Bax translocated to the surface of mitochondria forms homodimer or complex with Bak resulting in the release of cytochrome-C and caspase-9 activation (Skulachev, 1998). PUMA binds to Bcl-2 family pre-survival members such as Bcl-2 and Bcl-XL leads to Bax and Bak activation (Nakano and Vousden, 2001). Noxa induces apoptosis in a similar manner to PUMA and Bax. In addition to transcription dependent apoptosis induction, p53 also can

localize to mitochondria in cytoplasm and induce mitochondrial outer membrane permeabilization (Green and Kroemer, 2009).

Studies have shown that p53 induces ferroptosis by inhibiting SLC7A11 an important component of the cystine/glutamate antiporter, resulting in ROS induction (Jiang et al., 2015; Murphy, 2016). It has been shown that $p53^{3KR}$ an acetylation-defective mutant was unable to induce p53 mediated cell cycle arrest, senescence and apoptosis but still retains tumor suppression activity upon ROS upregulation.

p53 also influences metabolic pathway by regulating genes involved in respiratory and glycolytic pathways. For example, TIGAR, TP53-induced glycolysis and apoptosis regulator, inhibits glycolysis by decreasing the concentration of fructose-2,6-biphosphate. SCO2, synthesis of cytochrome c oxidase 2, promotes oxidative phosphorylation (Puzio-Kuter, 2011).

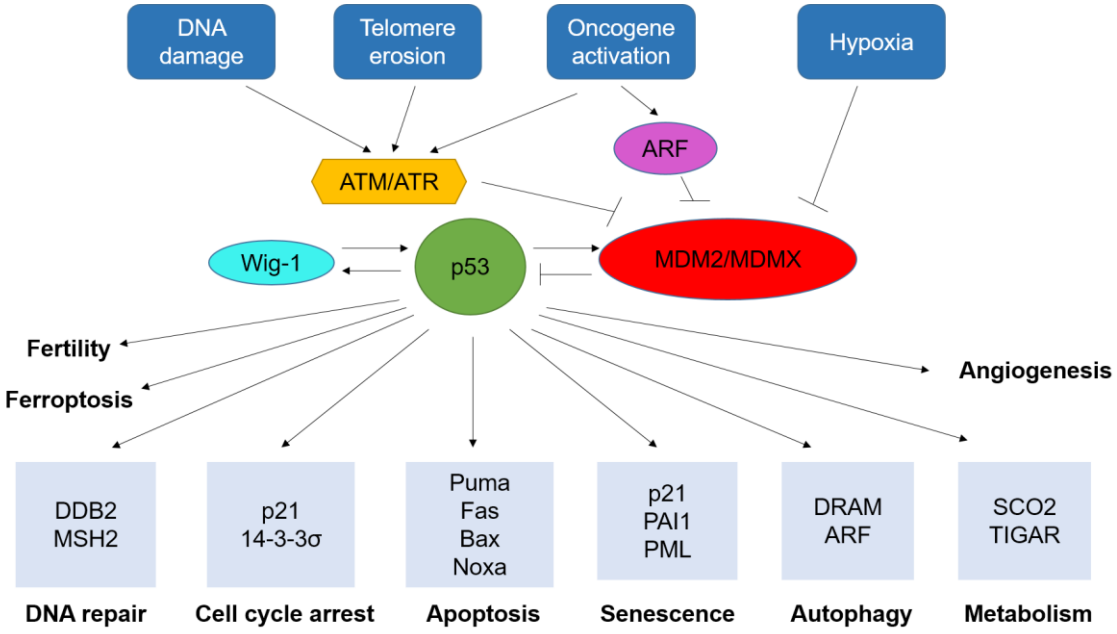

Figure 3. p53 regulation and signaling pathways.

1.3.4 p53 family member p63 and p73

Almost 20 years after p53 discovery, two other family members, p63 and p73 were identified. All three family members share a p63/p73 like common ancestor. The function of this ancestral gene is conserved during billion year's evolution, which is protecting the germline gametes. Phylogenetic analysis suggested p63, first identified in *C. elegans*, is the evolutionary oldest. p73 and p53 evolved from it. Like p53 structure, p63 and p73 also contain an N-terminal transactivation domain, a DNA binding domain and a C-terminal oligomerization domain. The DNA binding domain is highly conserved among all family members with more than 60% sequence identity. Because of two transcription start sites in the p63/p73 gene, two functional distinct protein isoforms TAp63/p73 and Δ-Np63/p73 are

produced. Studies have shown TAp63 and TAp73 can transactivate many p53 target genes and induce apoptosis (Shimada et al., 1999; Zhu et al., 1998). p63 and p73 have an extra sterile alpha motif (SAM) domain in the C-terminal, which be thought to mediate protein-protein interactions and inhibits transactivation activity of their TAD. Depletion of this region can enhance TAp63α and TAp73α transactivation activity (Ozaki et al., 1999; Suzuki et al., 2015).

TP63 is located on chromosome 3q27-29. p63 is rarely mutated but highly amplified and over expressed in human cancer. In addition to N-terminal isoforms, C-terminal alternative splicing leads to three additional isoforms α, β and γ. The ΔNp63α protein is predominantly expressed isoform in embryonic ectoderm and many epithelial tissues. It is crucial for proliferative potential of stem cells and epithelial tissue development. p63 null mice showed severer defect of in limb, craniofacial and epithelial development (Romano et al., 2012). Mutations in the DNA binding domain of p53 are strongly associated with Ectrodactyly, ectodermal dysplasia, and cleft lip/palate (EEC) syndrome. Mutations in p63 at sites 204, 279 and 304 in EEC syndrome patients correspond to hot spot mutation sites 175, 248 and 273 in p53 in human tumors (Celli et al., 1999). Moreover, it has been shown that p63 also has a key role in the control of aging process by downregulating sirt 1 (Sommer et al., 2006).

TP73 gene is located on chromosome 1p36. Like p63, only few p73 mutations were found in human cancer. p73 knockout mice do not develop tumors. However, high expression level of Δp73 has been found in many tumor types including neuroblastomas, melanomas, lung cancers and hepatocellular carcinomas (Stiewe and Putzer, 2002) and p73 overexpression is positively correlated with higher pathological stage and poor survival (Dominguez et al., 2001; Tannapfel et al., 1999). Studies have shown that p73 is enriched in the nervous system and plays an important role in neurological development (Talos et al., 2010). p73 deficient mice exhibit neurological and immunological defects (Yang et al., 2000).

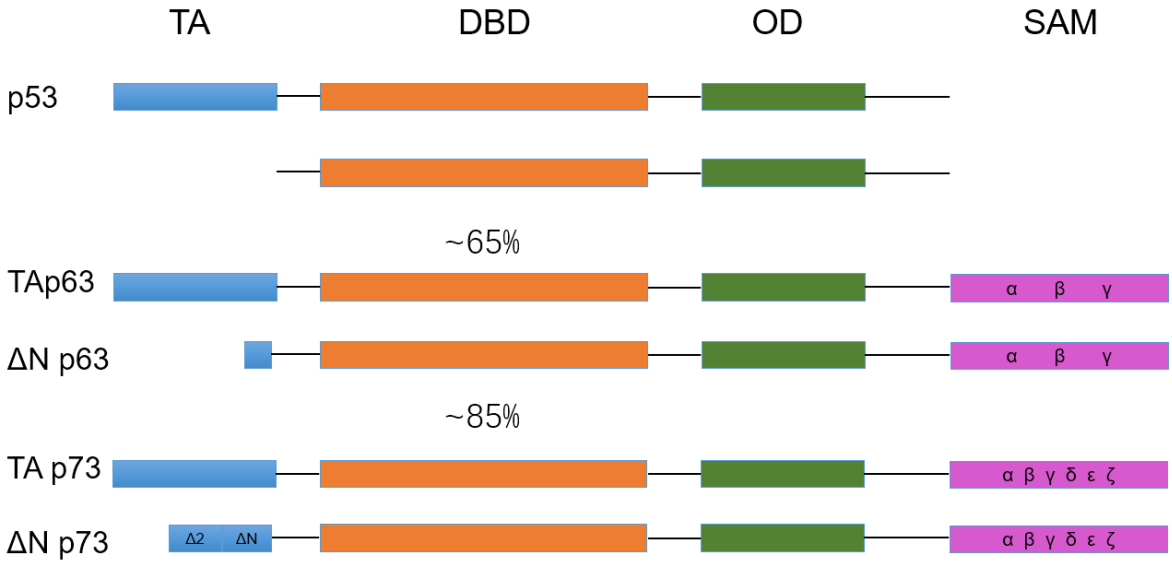

Figure 4. Schematic representation of the p53 family isoforms.

1.3.5 p53 inactivation and cancer

TP53 gene is the most frequently mutated gene in human cancer. Germline mutations within a defined region of p53 gene are associated with Li-Fraumeni syndrome and Li-Fraumeni like syndrome (Varley, 2003). Somatic mutations are found in most types of sporadic human cancer. The frequencies are varied from 10% to 90% depending on the type and stage of cancer (Figure. 5). The majority of p53 mutations are the missense mutation that accounts for 75% in human cancer. Frameshift and nonsense mutation each account for 10%. The rest 5% are mainly synonymous and splice site mutations. Approximately, 86% of p53 mutations occur in the conserved DNA binding domain (Olivier et al., 2010). Six sites (Arg-175, Gly-245, Arg-248, Arg-249, Arg-273 and Arg-282) represent almost 40% of p53 mutations and are defined as hot spot mutations (Olivier et al., 2002). p53 missense mutations are classified to two groups, DNA contact mutations and structural mutations. DNA contact mutations, just as its name implies, inactivate p53 by affecting the residues involved in DNA binding without altering global protein conformation. The examples of these substitutions are R273H, R248Q and R248W. Structural mutations such as R175H cause low stability and conformational change of p53. In addition to loss of functions (LOF), mutant p53 also gain some functions (GOF), by interacting with other proteins such as p63 and p73, thereby promoting tumor cell growth. Unlike most of other tumor suppressor genes that mutations in only one allele usually do not affect their activity, one allele mutation dramatically abrogates p53 functionality. The reason is wild-type p53 acts as a transcription factor as a homotetramer. In cells carrying mutations in one allele and the other allele remains wild type, two types of p53 form heterotetramers that usually are inactive.

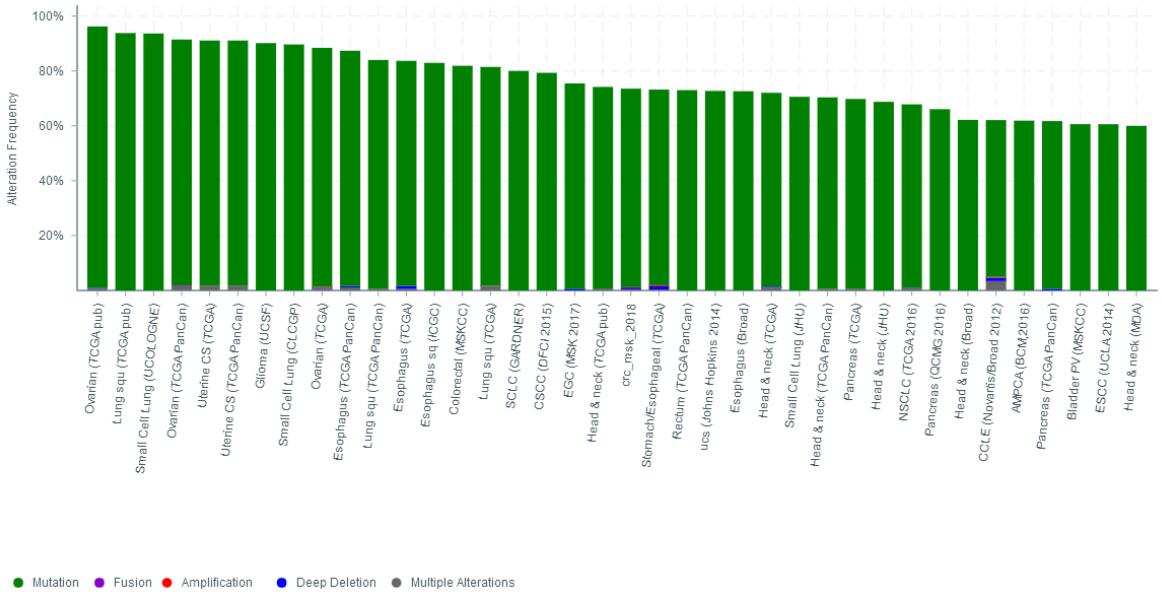

Figure 5. p53 alteration frequency in cancer.

In addition to mutation, p53 tumor suppressor functions also can be impaired by destabilization of p53 protein. As descripted above, MDM2 is a key p53 negative regulator binds to TAD of p53 that promotes p53 degradation. Amplification of MDM2 gene and over expression of MDM2 protein are features of many tumors (Gunther et al., 2000; Oliner et al., 1992). Viral proteins are also reported inactivate p53 by inducing p53 protein degradation. For instance, E6 encoded by human papilloma viruses (HPV) forms complex with p53 and targets it for proteasomal degradation (Scheffner et al., 1990; Thomas et al., 1999).

1.4 RESCUE OF WILD-TYPE P53 FUNCTION FOR CANCER THERAPY

1.4.1 Activation of wild-type p53

As described above, in many human tumors p53 is inactivated by MDM2/MDMX mediated p53 degradation. Thereby, it is logical to design drugs to reactivate p53 activity by stabilizing p53 through disrupting p53-MDM2 interaction. Nutlin, a specific MDM2 inhibitor, mimics three hydrophobic residues on p53 required for MDM2 binding. It blocks the binding of MDM2 to p53 resulting in p53 stabilization and activation (Vassilev et al., 2004). RG7112 binds MDM2 with a stronger affinity than Nutalin-3a and blocks p53-MDM2 interaction. It has been tested in several clinical trials (Kojima et al., 2016). Benzodiazepinediones disrupt p53-MDM2 interaction in a similar manner to nutlin (Koblish et al., 2006). RITA, a small molecule, has been identified that prevents p53–MDM2 interaction by inducing p53 conformational change through binding to the N-terminal of p53 (Issaeva et al., 2004). Notably, RITA also induces the proteasome-dependent degradation of MDMX (Spinnler et al., 2011).

1.4.2 Reactivation of mutant p53

The high frequency and high expression level of mutant p53 in human tumors makes it an attractive target for cancer therapy. The strategy is to restore and stabilize the native conformation of p53 DNA binding domain for specific DNA binding thereby inducing p53 target gene activation to induce tumor cell apoptosis. Studies have shown p53 can be stabilized by introducing a second mutation (Blandino et al., 2012). CDB3, a small peptide derived from a p53 binding protein, binds to native but not denatured p53 core domain, shift the equilibrium between folded and unfolded protein towards the folded state, and restores specific DNA binding for mutant p53 (Friedler et al., 2002). Many compounds have been identified by chemical library screening, molecular modeling or rational drug design that can restore wild type functions to mutant p53 such as, CP-31398， MIRA-1, STIMA-1, PK11007, ZMC1, stictic acid and PRIMA-1/APR-246 (Bykov et al., 2018).

CP-31398 was identified by screening a library of synthetic compounds that stabilizes wild-type p53 conformation and restores wild-type p53 functions to mutant p53 (Foster et al., 1999; Wischhusen et al., 2003). In vivo study has shown that CP-31398 prevents growth and invasion of bladder tumors in transgenic UPII-SV40T mice (Madka et al., 2013). CP-31398

has Michael acceptor activity. N-acetylcysteine (NAC) partially inhibits CP-31398 induced growth suppression in H1299His175 cells (Zache et al., 2008).

MIRA-1 and STIMA-1 were identified by screening molecular libraries that target mutant p53 expressing cells and induce p53 target gene activation (Bykov et al., 2005a; Zache et al., 2008). Both of them are Michael acceptors participate in reactions of nucleophilic addition, prone to react with free thiol groups in the cell. It has been shown that MIRA-1 also induces p53-independent growth suppression by caspase 9 activation (Bou-Hanna et al., 2015).

PK11007, one of 2-sulfonylpyrimidines, is a thiol alkylator that has anti-tumor activity in many tumor cell lines. It can act in both p53-dependent and p53-independent manners (GSH depletion) (Bauer et al., 2016). Cys182 and Cys277 are crucial for PK11007 mediated mutant p53 reactivation.

ZMC1(NSC319726) as a Zn^{2+} chelating compound buffers intracellular Zn^{2+} to an optimal concentration for binding to p53, which is crucial for wild-type p53 structural stability. It induces PAb1260 (wild-type p53 conformation specific antibody) epitope and p53 dependent apoptosis in H1299His175 cells (Yu et al., 2014; Yu et al., 2012). There is an imine group which can react with thiol in ZMC1 may contribute to its biological activity.

Stictic acid was identified by *in silico* screening that binds to the binding pocket between loop L1 and sheet S3 of the p53 core domain. It thermostabilizes R175H and G254S p53 mutants and induces p53 target gene p21 expression in a dose dependent manner (Wassman et al., 2013).

PRIMA-1 was identified by screening the NCI library of low molecular weight compounds that suppresses tumor cell growth in a mutant p53 dependent manner (Bykov et al., 2002). Later on, we found the methylated analog PRIMA-1^{met}/APR-246 is more potent. *In vivo* studies have shown that PRIMA-1 and APR-246 inhibit growth of tumor xenografts expressing mutant p53 in SCID mice (Bykov et al., 2002; Zandi et al., 2011). Recent studies have shown APR-246 also induces p53-independent cell death mainly by regulating ROS. APR-246 inhibits Thioredoxin reductase (TrxR1) presumably by covalent binding to a selenocysteine residue in TrxR1 and contributes to ROS production (Peng et al., 2013). MQ binds to cysteine in glutathione (GSH) and forms GS-MQ, which significantly reduces the activity of GSH results in ROS accumulation (Liu et al., 2017; Tessoulin et al., 2014). APR-246 showed synergistic effects with conventional chemotherapeutic drugs such as cisplatin, doxorubicin were observed both *in vitro* and *in vivo* (Bykov et al., 2005b; Liu et al., 2015). A phase I/IIa clinical trial of APR-246 in patients with leukaemias, lymphomas or prostate cancer is completed (Lehmann et al., 2012). This study defined the maximum tolerated dose (MTD) as 60mg/kg and showed only minor and transient side effects, including dizziness, fatigue, headache, nausea, and confusion. Currently, APR-246 has been tested in a phase II clinical trial in patients with high-grade serious ovarian cancer (HGSOC), which is characterized by p53 mutations. Furthermore, a clinical trial in Oesophageal cancer patients is ongoing. Studies of APR-

246 in combination with other drugs are also under way for example, APR-246 combined with Dabrafenib in melanoma, with Pegylated liposomal Doxorubicin Hydrochloride (PLD) in HGSOC, with Azacitidine in patients with Myelodysplastic syndrome, acute myeloid leukemia, myeloproliferative neoplasm or chronic myelomonocytic leukemia(clinicaltrials.gov). Both PRIMA-1 and APR-246 convert to MQ, an electrophile has Michael acceptor activity. The electron-withdrawing carbonyl group in MQ makes the carbon-carbon double bond highly reactive that is prone to attack thiols. Previous study has shown MQ covalently binds to the cysteines in p53 core domain (Lambert et al., 2009). Cysteine to alanine substitution at position 124 in p53 core domain abolishes APR-246 mediated mutant p53 reactivation (Wassman et al., 2013).

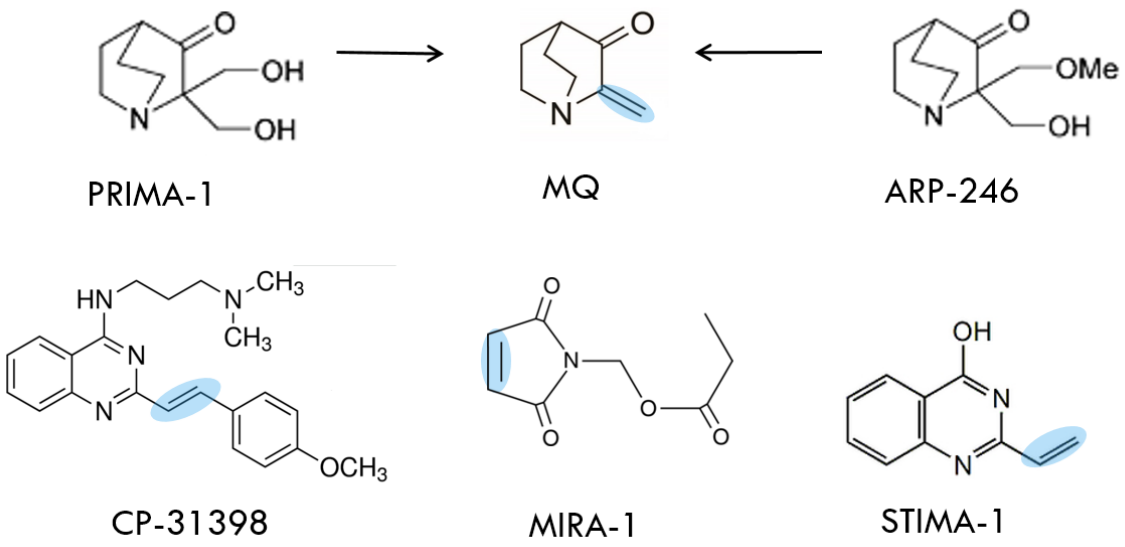

Figure 6. Chemical structure of mutant p53 reactivating compounds. Blue indicates the thiol reacting sites.

2 AIMS OF THE THESIS

The general aim of this thesis is to improve drug design and development in cancer treatment by studying the structure of p53 family members during evolution and the molecular mechanisms of mutant p53 reactivation.

Specific aims

Paper I

To investigate whether APR-246 reactivates mutant p63 in keratinocytes derived from EEC syndrome patients.

Paper II

To elucidate the mechanism of APR-246/MQ-mediated mutant p53 reactivation.

Paper III

To understand the relationship between thiol reactivity, mutant p53 reactivation and tumor cell death.

Paper IV

To reach a better understanding of the structure of p53 and the factors govern its stability during the evolution.

3 RESULTS AND DISCUSSIONS

3.1 PAPER I

APR-246/PRIMA-1MET rescues epidermal differentiation in skin keratinocytes derived from EEC syndrome patients with p63 mutations

p63 shares high sequence and structure similarities with p53. p63 lacks mutation in cancer development. Instead, mutations of p63 cause severe defects in epidermal, craniofacial and limb development such as ectrodactyly, ectodermal dysplasia, and cleft lip/palate (EEC) syndrome. Some p63 mutations associated with EEC syndrome R204W, R279H and R304W correspond to hot mutations R175H, R248 W and R273H inp53 respectively. Previously we have shown that PRIMA-1/APR-246 restores wild-type p53 activities to mutant p53 to induce apoptosis in human tumor cells. In this study, we want to investigate whether APR-246 reactivates mutant p63 in human keratinocytes derived from EEC syndrome patients.

We established keratinocyte models derived from EEC syndrome patients carrying p63 mutations (R204W, R279H or R304W) in the DNA binding domain of p63. By analyzing the expression profile, we found the 328 genes showed significant and consistent changes in all mutant cells and identified 208 of them are potential p63 target genes. ADH7 is one of these genes that the expression was almost lost in cells with p63 mutations in the DNA binding domain. We observed that the morphological features of cells with R204W and R304W mutations are differ from wild-type p63 cells. Compared to wt cells, mutant p63 cells are larger, flatter under proliferative condition. After differentiation, wt cells started forming multilayers but mutant p63 cells were either detached or maintained as a single layer.

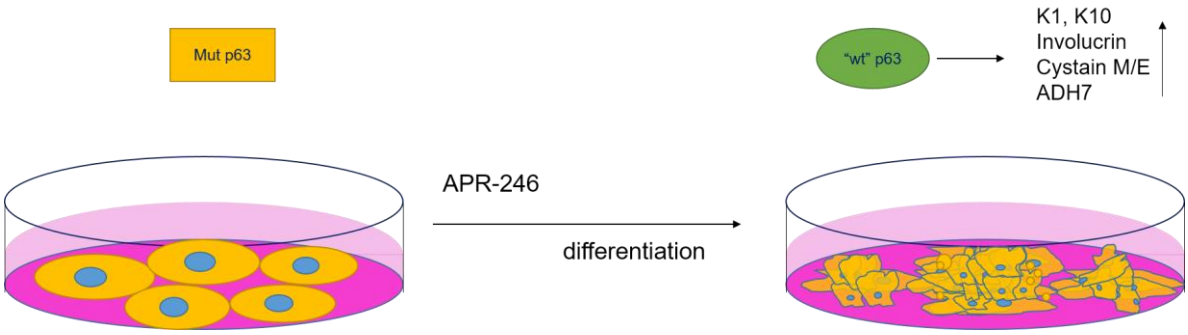

Figure 7. APR-246 restores morphology of keratinocytes with mutant p63 and reactivates its target genes.

Does mutant p53 reactivating compound APR-246 restore wild-type functions to mutant p63 in EEC patients? To answer this question, we treated mutant p63 keratinocytes in 2D and 3D cultures with APR-246. We observed that treatment partially rescued wt morphology in mutant p63 cells. Furthermore, we observed induction of markers in epidermal differentiation and stratification, such as K1 and K10 in epithelial, involucrin in the spinous layer, TGM, CysME, LCE2 and Filaggrin in the granular layer, after APR-246 treatment. Moreover, p63

target gene ADH7, GPX2 and Claudin-1 were also induced in both protein and mRNA level upon APR-246 treatment. All our observations suggest that APR-246 recues human skin epidermal differentiation by restoring wild-type functions to mutant p63.

Our study may open new insights for screening and designing novel drugs for pharmacological treatments for patients with mutant p63 associated developmental disorders as wells as other mutant p63 related diseases.

3.2 PAPER II

APR-246 reactivates mutant p53 by targeting cysteines 124 and 277

We have shown than the conversion products of PRIMA-1 bind covalently to cysteines in the p53 core domain. However, which cysteines among 10 cysteines in p53 core domain are the exact targets for MQ remains unknown.

Firstly, we found MQ bound to wild-type p53 core domain as well as R175H and R273H mutants in a dose dependent manner. However, the inactive analog MQ-H was unable to bind any of them. In accordance with the binding results, we showed MQ but not MQ-H enhanced the thermostabilities of wt and mutant p53 core domains by both differential scanning fluorimetry (DSF) and circular dichroism (CD). To determine if APR-246/MQ can refold mutant p53 in cellular environment we treated TOV-112D ovarian carcinoma cells which carrying R175H structure mutant with APR-246/MQ. We observed that APR-246/MQ induced PAb1620, a "wt" like structure specific p53 antibody, positive staining and decreased mutant p53 structure specific antibody HO3.5 staining. However, MQ-H failed in inducing any change in either PAb1620 or HO3.5 staining. All these results indicate that Michael acceptor activity of MQ is essential for mutant p53 reactivation. Subsequently we identified Cys277 as a prime binding site for MQ by performing mass spectrometry with MQ treated p53 core domain proteins contain cysteine (Cys) to alanine (Ala) substitution at sites 124, 182, 249 or 277. Later on, we found that Cys277 is also essential for MQ mediated thermostailization of p53 core domain by performing DSF with these core domains

To understand the role of cysteine residues in mutant p53 reactivation in living tumor cells, we transfected human lung carcinoma H1299 cells with R175H, R175HC124A, R175H C277A or R175H-C124A-C277A vectors. After APR-246 treatment, we found that APR-246 induced Annexin V positive staining in cells transfected R175H construct but not cells with C124A or C277A mutations indicated C124A and C277A abolished APR-246 induced apoptosis. Furthermore, we want to know if the C124A and C277A can affect APR-246 induced p53 target gene activation. We found cells transfected with constructs contain C124A or C277A mutations showed significantly reduction on the expression of p21, Fas and Bax, which is consistent with the failure of apoptosis induction. However, the abolishment of apoptosis and p53 target gene activation is plausible due to the damage of p53 activities by C124A or C277A substitution. To determine this, we transfected H1299 cells with wt, C124A, C277A, R175H or R273H constructs and assessed apoptosis and p21 induction. We observed apoptosis as well as p21 induction in cells expressing wt, C124A and C277A p53 but not in cells with R175H and R273H mutants indicate C124A and C277A do not inactivate p53.

In conclusion, the Michael acceptor activity is essential for MQ to bind, stabilize and refold p53. Cys277 as a prime target for MQ is critical for p53 thermostabilization. Both Cys124 and Cys277 are required for APR-246/MQ mediated MQ induced mutant p53 reactivation in living tumor cells.

3.3 PAPER III

Role of thiol reactivity for targeting mutant p53

Many identified mutant p53 reactivation compounds such as APR-246, MIRA-1 and PK11007 share one common feature: having the ability to react with thiols. Is there any correlation between thiol-reactivity and mutant p53 reactivation?

To address this question, we selected potential thiol reactive compounds in the National Cancer Institute (NCI; Bethesda, MD) library based on the chemical structure (Michael acceptor, imine, primary alcohol and aldehyde) that showed biological activity ($IC50<=10^{-4}M$) in at least 3 cell lines in NCI screening. Compounds showed top p53 selectivity, IC50wt/ IC50 mutant >=1,4 in all five selected tumor types (leukemia, NSCLC, melanoma, ovarian cancer and renal cancer) which contain at least 2 wt p53 expressing cell lines and at least 2 mutant p53 expressing cell liens, were selected for subsequently studies.

We performed experiments with H1299/H1299His175 and Saos-2/Saos2His273 cell systems to study the ability of each compound to react with thiols and to induce growth suppression, subG1 population, cell cycle arrest, mutant p53 refolding, thermostabilization and ROS. A complex data matrix was generated from those experiments. In order to get meaningful information from the data matrix, we performed multivariate analysis. We identified four important factors that explained around 50% of the variability of the data by employing standard extraction method *communalities = multiple R2* with Kaiser Criterion. Subsequently, we performed so called K-mean clustering technique to stratify all the compounds into three clusters: Cluster I compounds are characterized as toxic thiol reagents; Cluster II compounds are p53 refolders and cluster III are thermostabilizers. To investigate the relationships between the functional effects and chemical functional groups we did correspondence analysis. We found that compounds with Michael acceptor group either alone or together with imine are prone to react with thiols and have high toxicity. p53 refolders are more likely to have alcohol groups either alone or combined with imine or combined with imine and Michael acceptor functionalities. Aldehyde or imine groups or Michael acceptor combined with either aldehyde or alcohol functionality are more likely to contribute to thermostabilization.

In our study, we found the degree of thiol reactivity is critical for mutant p53 targeting compounds. Mild thiol reactivity stimulates mutant p53 refolding but too high reactivity often leads to general toxicity. Interestingly, we found that compounds with one functional group alone or combined with other groups can belong to different clusters for example compounds with alcohol groups belong to p53 refolders but compounds with alcohol combined with Michael acceptor are more like to be thermostabilizers. A molecule can presumably gain desired p53 dependent biophysical or biological effects by judicious design of thiol-reactive functionalities. Our study sheds light on a new perspective for developing more potent mutant p53 targeting drugs.

3.4 PAPER IV

The DNA-binding domain of a p53 homolog from the hydrothermal annelid *Alvinella pompejana*: protein stability and evolutionary history of the p53 family

In this study, we obtained a transcript containing p53-like DBD by performing a BLAST search of the *Alvinella pompejana* expressed sequence tag (EST) database. The genome sequence of this worm is not available yet, but analysis of published genomes of two other annelid worms, the marine polychaete *Capitella teleta* and the freshwater leech *Helobdella robusta* suggests the presence of a single p53 family gene in *Alvinella*. *Capitella* and *Alvinella* belong to the same class (polychaetes worms). Prediction of *Capitella* p53 homolog suggests it contains a p63 and p73 characteristic SAM domain. Sequence alignment of *Alvinella* p53 DBD showed that it has high sequence identity with the DBD of human p53 family members: p53 (47%), p63 (58%) and p73 (56%). The conservation is even higher between *Alvinella* and *Capitella*, more than 80%. By homology modeling of *Alvinella* DBD, we found that the human p53 corresponding residues in the zinc binding sites (Cys176, Cys238, Cys242 and His176) and the key DNA binding sites (Arg282, Arg273 and 280) are conserved in *Alvinella*.

A double salt bridge formed by Glu180 and Arg181 between two human p53 DBDs is a typical motif associated with p53 binding cooperativity. This motif is a typical feature of vertebrate p53 except rainbow trout. However, it is missing in human p63/p73, *Alvinella* homolog and other invertebrate p53 homologs. Evolutionary study showed the first appearance of this motif is in cartilaginous fishes in the evolutionary tree of p53 family proteins. Altogether indicate the double salt bridge is special event of p53 evolution appears in early stage of vertebrate.

To investigate protein thermotability, we performed DSF and DSC with the *Alvinella* DBD and selected vertebrate p53 variant. We found the *Alvinella* DBD was the most stable one among all tested proteins followed by the DBD of chicken. Their Tms were much higher than the Tm of the human p53 DBD. However, the DBD of cold-blood frog was less stable than the human DBD. The melting temperature of DBD from rainbow trout, a cold-water animal, was even lower. The correlation between protein thermotability and the organismal and living environmental temperature indicates that p53 has evolved to be more suitable for organisms during evolution.

Why is *Alivnella* DBD so stable? One possibility could be the high sequence conservation with more stable homolog p63, which has an optimal packing of the hydrophobic core. It has been shown that packing of hydrophobic core correlates protein stability. F270L mutation in the hydrophobic core of the human p53 DBD reduces thermotability and leads to protein unfolding and aggregation. *Alivnella* DBD has a similar large to small substitution F270V. However, a neighbor small to large substitution I255F compensates it that maintains the optimal packing of hydrophobic core. This inverted packing pattern is found in most of invertebrate p53 family sequences, however, all extant vertebrates share the same 255/270

packing pattern found in human p53. Moreover, it has been reported that human p53 DBD is stabilized by removing the non-saturated hydrogen bond between Y236 and T253. Substitutions with equivalent hydrophobic residues were found in more stabled p63/p73 and *Alvinella* DBD. Furthermore, it have been reported that a second site N239Y mutation stabilizes human p53 DBD, Phenylalanine at the same position in *Avinella* DBD may have similar packing interactions as tyrosine which stabilizes human N239 mutant.

In summary, our structural and phylogenetic analysis suggests that the *Alvinella* p53 homolog is more p63/p73 like. The modeling study indicates a series of repacking features of the hydrophobic core are associated with its high thermotability. By understanding the structure of p53 family members and the factors govern their stabilities in evolution may open new insights for us to develop mutant p53 targeting drugs for cancer therapy.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

Transcription factor p53 plays key roles in tumor suppression and regulates various signaling pathways involved in this process. p53 mutations are reported in almost all cancer types. The mutation frequencies vary from 10% to almost 100%, on average 50%. Therefore, restoration wild-type p53 functions to mutant p53 becomes an attractive strategy for cancer treatment since the first mutant p53 reactivating compound CP-31398 was identified in 1999.

In order to design and develop novel and more potent mutant p53 targeting drug we need to understand more about p53, including the change of its structural features between wild type and mutant states. People believe mutations inactivate p53 by unfolding its structure and eventually disrupting its ability to bind to DNA. Many p53 cancer mutations are temperature sensitive can be rescued by simply low down the temperature. Interestingly, it has been shown that the *xenopus* p53 protein behaves like the temperature sensitive p53 cancer mutants. Further studies have shown the thermostability of p53 DBD has correlation with the body and living environmental temperature of the organism. *Avinella* is a thermophile. It is interesting to study the thermostability of p53 in this worm. In paper IV, we found that p53 homolog in *Avinella* is more p63/p73 like. The thermostability of its DBD is much higher than human p53 DBD but lower than human p63 DBD. To understand what factors lead to its high stability we compared its sequence with human p53, p63 and p73. We found *Avinella* protein has several packing features of the hydrophobic core such as, removing non-saturated hydrogen bond, residues form stabilizing packing interaction, are similar to p63, p73 and stabilized p53 mutant. It might be feasible to design drugs to govern p53 thermostability by modulating the packing of its hydrophobic core.

p53 and p63 are highly conserved in the DBD. It is logical to study whether mutant p53 reactivating compound, APR-246 can also reactivate mutant p63. Previous study has shown APR-246 can restore wild-type function to mutant TAp63γ in human tumor cells. However, p63 is rarely mutated in human cancer. Mutations in p63 are highly associated with many human developmental defect syndromes such as, EEC and AEC. In paper I, we found that APR-246 could reactivate R204W and R304W mutants and induce p63 target gene expression. In addition, we observed APR-246 rescues the morphology of human keratinocytes carrying mutant p53 derived from EEC syndrome patients. However, the mechanism of mutant p63 reactivating is unclear. It will be interesting to study whether mutations in p63 lead to protein destabilization and unfolding. Whether APR-246 binds to p53, if so, where does it bind to p63? Does APR-246 stabilize and refold mutant p63? By studying there questions, we will have a better understanding about APR-246 and inspire us how it reactivates mutant p53.

Our previous studies have shown, PRIMA-1 and APR-246 convert to MQ, the active compound, covalently binds to cysteines in p53 core domain. However, which cysteines are the targets remains unclear. In paper III, we showed that the Michael acceptor activity is essential for the binding of MQ to p53. Lack of this activity MQ failed to stabilize and refold p53 protein. MQ is an electrophile prone to react with nucleophiles. Thereby thiol group in cysteine is a prime target. Based on the crystal structure of p53, we know that p53 has 10 cysteines with various solvent accessibility in the core domain. Cys277 and Cys182 are on the surface of p53, which could be easily reached by MQ, followed by Cys229. Cys124 has been reported plays a key role in APR-246 mediated mutant p53 reactivation. By Cys to Ala substitution at these sites, we found that Cys277 is a prime binding site for MQ and it is important for MQ mediated thermostabilization of p53 core domain. Of course cysteines are buried in the hydrophobic core can be exposed in certain condition. We have shown that all ten cysteines can be reached when the concentration of PRIMA-1 is up to 5mM, however, core domains with C277A substitution were not able to be stabilized by increasing MQ concentration indicating Cys277 is crucial for MQ mediated p53 thermostabilization. Subsequently we studied the role of cysteine Cys124 and Cys277 on mutant p53 reactivation by MQ in living tumor cell. The side chain of cysteine is much bigger than alanine. Does this substitution have any effect on wild type p53 activity? We observed C124A and C277A mutations did not affect wild-type p53 tumor suppressor activity but abolished APR-246 induced apoptosis and p53 target gene activation, indicating Cys124 and Cys277 are important for APR-246 mediated tumor suppression. Although it has been shown that APR-246 induces cell death in FLO-1 cells, carrying C277F, it mainly due to inhibition of SLC7A11 resulting in ROS induced cell death.

In paper II, we found that the ability of MQ to react with cysteines is needed for mutant p53 reactivation. Interestingly, several other mutant p53 targeting compounds, CP31398, MIRA-1, STIMA-1, PK11077, are also have thiol reactivity. In paper IV, we studied the relationship between thiol reactivity and mutant p53 reactivation activity. We found that strong thiol reactivity leads to toxicity and only mild activity is required for mutant p53 reactivation. Various chemical functional groups are associated with different properties for mutant p53 reactivation. Desired biological and biophysical goals for mutant p53 reactivation are possible to be achieved by the elaborate design of thiol-reactive functional groups.

Altogether, a better understanding of p53 structure and exact mechanisms of current mutant p53 reactivating molecules will inspire us and give us new insight to facilitate the design of more potent p53-selective compounds and the development of rational drugs for cancer treatment.

5 ACKNOWLEDGEMENTS

During my whole PhD studying time there are so many people supported me, helped me and accompanied me. I would like to extend my sincerest gratitude to all of you. Without you, I would not have such a great time in my PhD journey. Many good memories during these years will accompany me all my life. Thank you!

Firstly, I would like to thank my main supervisor **Klas Wiman** for giving me the opportunity to start my PhD in such a fantastic group. I feel extremely lucky to be your student. You are a nice, knowledgeable and encouraging person. You are a great scientist. Your enthusiasm about science inspired me through my whole PhD study. Your optimism influenced me and made me have the courage to overcome all the difficulties. With your help and supervision, I gradually become a mature and independent researcher. I am and will be very proud of being your student.

I also would like to thank my co-supervisor **Vladimir Bykov**. Thank you for your supervision during these years. I learnt a lot from you. You are such a kind person. I can always get help from you whenever I come to you. I was deeply impressed by your great knowledge. You seems know everything and can easily solve all kinds of questions. You are not only a supervisor for me but also a friend, a mentor. Your encouragement and concerns helped me out of trough. We had lots of discussions not only about science but also all other aspects such as food, traveling, Kong Fu, etc. I really enjoyed a lot.

I want to thank **Thierry Soussi** for giving me the opportunity to collaborate with you. It is realty good to have you for giving tweeters during our lab meeting. Your great knowledge in p53 field impressed a lot and broadened my horizon. You are a kind and humorous person in your daily life. However, you become so strict when you are doing science. Your attitude for doing science will affect me in my future life.

Thank you **Joanna Zawacka-Pankau** for teaching me all the techniques during my PhD study. We had very hard times but finally we overcame that and got our paper published. Thanks for all the discussions and suggestions for our paper.

Thanks to all the current and previous members in our group. **Susanne**, for the help from you during my PhD study. It is good to have you in our lab. You make our lab works in order. **Lidi, Mei**, **Emarn,** for all your help in the lab and the nice time we had dinner and salsa dancing together. It is my honor that having you during my PhD. **Sophia** and **Sofi** for all your suggestions and help. We had a wonderful trip in Melbourne. **Julie, Angelos and Mireia** for all your support. We had lots of fun during our nice lunchtime. Good luck for your future. **Cinzia,** for all nice fika you made. **Fredrik, Jin, Anna, Salah, Sofia, Nina, Helene,** for being colleagues with you and fun time we had together.

I would like to thank all people from CCK, **Yingbo,** for all exciting discussions with you and all the help from you. I had a lot of fun when we had dinner together in your place. **Xiaonan Zhang & Fan Zou,** for many good food and happy time. You are such nice people and always give me positive energy. **Yuanjun Ma and Changwu Lu**, for a great time in Gran Canaria. **Na Wang, Kelin Jia**, for nice trip to Kiruna and all the good Sichuan food. **Dudi, Roger** for all the nice time discussed with you and good time we had dinner together. **Ran Ma & XinSong Chen** for great time we played games together. **Min Guo, Rong Yu, Tong Liu, Huiyuan Zheng, Hong Xie, Zhiwei Liu, Yali Miao, Zhuochun Peng, Xin Wang, Xianli Shen, Limin Ma, Yuanyuan Zhang, Muyi Yang, Chao Sun, Shuo Liang** for all your help and company. **Chen Yang, Lu Wang, Yi Chen, Hao Shi, Wenkuan Huang, Jiwei Gao, Dawei Song, Yaxuan Liu, Nini Mu, Weng-Onn Lui, Soniya Dhanjal, Satendra Kumar, Patrick, Cristin,** for all nice time with you, wish all of you good luck.

My friends from other departments of KI, **Yu qian,** for all you help with fixing my car. I can see you really like machine. **Rui Wang,** for all your help and nice Shanxi dinner. **Fei Yang** for teaching me how to snowboard. **Ting Zhuang, Jian Zhu, Bingnan Li, Xiaolu Zhang, Tiantian Liu, Tianwei Gu, Xiaoyuan Ren, Jiangrong Wang,** for all the good time we spent together.

Shanshan Xie, Jinsong Wang, Min lei, Kjell, for your carrying. We had a lot of fun together.

My friends from Beijing Jiaotong University. **Juan Du,** for all your help. Without that I may never come to Sweden to have my PhD. **Hongya Han, Xinyan Miao,** we knew each other for many years, thank you for all the help and all the nice time we spent together. **Jilin Zhang, Shaohua Xu, Lu Zhang,** for the good party time and nice dinners, wish all the best to you.

My friends play badminton together. **Feng Wan,** it is good to be friend with you. We had a lot of fun together. Hope you will be a great director. Let's party. **Peng Zhang** for your good food and playing football together. I will invite you for Beijing duck and Chinese alcohol. Don't waste again. **Chengjun Sun,** for all the good dinner you cooked. **Genping Huang, Hongyan Zou, Jiangnan Luo, Bo Cao, Wei Hu,** for great drinking days. **Qiong Zhang, Michael Yang, Miky Yang, Tongmei Wang, Jing Guo, Jie song, Peiliang Chang, Xuejin Liu,** I really enjoyed that time we play badminton together. Thanks for all your company and all the nice dinners.

Thanks to all friends, we played poker together. Especially, **Kai Du,** you are a great organizer. Thanks for inviting all of us to your home every Friday night. **Qin Xiao, Jianren Song, Na Guan, Yuning Zhang, Menghan Gao, Zheng Chang, Ci Song, Jianwei Zhu, Huan Song, Chen Suo, Jiaqi Huang, Yixin Wang, Dong Yang, Xinming Wang, Celine,** for all the memorable poker night. Together with you, I never felt lonely.

Xuan yang, Simba, Hongmei yan, Meiying Sun, Chencheng Zhang, Molan Li, Minyue Li, Leo, Sisi Huang, Jidong Tan, Ying Liu, Jisheng Wang, Jenny Huang, Xiaodong Zou, Yaping Qu, for having fun in the choir. We had many great performances. Cheers!

Wang Zhang, Jing Wang, Dawei Zhang, Ying Liu, Zhenhua Shan, Xiaomei Huang, you are such nice and self-giving people. I learnt a lot from you. Best wishes to all of you.

Yihai Cao, Peiqun Ye, Bin Zhou, Hai Bo, Qiang Chen, Jiang Yu, Qing Zhang, Qilin Fu, Yanhao Su, Yan Wang, Hong Zhang, Xuechao Wei and all other members from our association. It is my honor to be a member of such nice team. I hope everything goes well to our association.

Thanks to all my friends I may not mentioned here. You altogether made me a great time during my PhD.

致我亲爱的家人 （To my dearest family）

非常感谢我的**爸爸妈妈**，感谢你们的抚养和无私的爱。你们永远是我坚强的后盾和温暖的港湾，我知道无论我面临什么样的困难你们都会义无反顾的支持和鼓励我。 我爱你们。

奶奶您是我们家的顶梁柱，是您把我们一大家人团结在了一起，给我们一个温暖的大家庭。家和万事兴，希望您健康长寿。感谢我们**兄弟们**，我们虽然是表兄弟，但是我们胜似亲兄弟。感谢我**所有的亲人们**，能和你们成为一家人真好。

感谢我的妻子**胡芳的父母和哥嫂**。感谢岳父岳母生养了胡芳，让我娶到这么好的妻子。感谢哥哥和嫂子对岳父岳母的照顾，让胡芳能安心的在国外陪我。

特别感谢我的妻子**胡芳**，我们的结合是上天的安排，是缘分把你从意大利带到了我的身边。你的出现让我不再漂泊，有了安定的感觉。你细心的照顾和你的理解让我有了更好的环境来完成我的学业。非常感谢你为我们家增添了新的人口，**宇瑞**的出生让我们家里更加兴旺，让我觉得我自己现在责任更重了，但更有为了我们生活更加美满而奋斗的动力。

米豆，感谢你的到来，我会和妈妈一起好好爱你，陪你成长。 我们是最幸福的一家人！

6 REFERENCES

Abida, W.M., and Gu, W. (2008). p53-dependent and p53-independent activation of autophagy by ARF. Cancer Res *68*, 352-357.

Baptiste, N., Friedlander, P., Chen, X.B., and Prives, C. (2002). The proline-rich domain of p53 is required for cooperation with anti-neoplastic agents to promote apoptosis of tumor cells. Oncogene *21*, 9-21.

Bauer, M.R., Joerger, A.C., and Fersht, A.R. (2016). 2-Sulfonylpyrimidines: Mild alkylating agents with anticancer activity toward p53-compromised cells. P Natl Acad Sci USA *113*, E5271-E5280.

Beausejour, C.M., Krtolica, A., Galimi, F., Narita, M., Lowe, S.W., Yaswen, P., and Campisi, J. (2003). Reversal of human cellular senescence: roles of the p53 and p16 pathways. Embo J *22*, 4212-4222.

Blandino, G., Deppert, W., Hainaut, P., Levine, A., Lozano, G., Olivier, M., Rotter, V., Wiman, K., and Oren, M. (2012). Mutant p53 protein, master regulator of human malignancies: a report on the fifth Mutant p53 Workshop. Cell Death Differ *19*, 180-183.

Bochkareva, E., Kaustov, L., Ayed, A., Yi, G.S., Lu, Y., Pineda-Lucena, A., Liao, J.C., Okorokov, A.L., Milner, J., Arrowsmith, C.H.*, et al.* (2005). Single-stranded DNA mimicry in the p53 transactivation domain interaction with replication protein A. Proc Natl Acad Sci U S A *102*, 15412-15417.

Bode, A.M., and Dong, Z. (2004). Post-translational modification of p53 in tumorigenesis. Nat Rev Cancer *4*, 793-805.

Bottger, A., Bottger, V., GarciaEcheverria, C., Chene, P., Hochkeppel, H.K., Sampson, W., Ang, K., Howard, S.F., Picksley, S.M., and Lane, D.P. (1997). Molecular characterization of the hdm2-p53 interaction. J Mol Biol *269*, 744-756.

Bou-Hanna, C., Jarry, A., Lode, L., Schmitz, I., Schulze-Osthoff, K., Kury, S., Bezieau, S., Mosnier, J.F., and Laboisse, C.L. (2015). Acute cytotoxicity of MIRA-1/NSC19630, a mutant p53-reactivating small molecule, against human normal and cancer cells via a caspase-9-dependent apoptosis. Cancer Lett *359*, 211-217.

Bykov, V.J.N., Eriksson, S.E., Bianchi, J., and Wiman, K.G. (2018). Targeting mutant p53 for efficient cancer therapy. Nat Rev Cancer *18*, 89-102.

Bykov, V.J.N., Issaeva, N., Shilov, A., Hultcrantz, M., Pugacheva, E., Chumakov, P., Bergman, J., Wiman, K.G., and Selivanova, G. (2002). Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. Nat Med *8*, 282-288.

Bykov, V.J.N., Issaeva, N., Zache, N., Shilov, A., Hultcrantz, M., Bergman, J., Selivanova, G., and Wiman, K.G. (2005a). Reactivation of mutant p53 and induction of apoptosis in human tumor cells by maleimide analogs. J Biol Chem *280*, 30384-30391.

Bykov, V.J.N., Zache, N., Stridh, H., Westman, J., Bergman, J., Selivanova, G., and Wiman, K.G. (2005b). PRIMA-1(MET) synergizes with cisplatin to induce tumor cell apoptosis. Oncogene *24*, 3484-3491.

Campisi, J., and di Fagagna, F.D. (2007). Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Bio *8*, 729-740.

Celli, J., Duijf, P., Hamel, B.C., Bamshad, M., Kramer, B., Smits, A.P., Newbury-Ecob, R., Hennekam, R.C., Van Buggenhout, G., van Haeringen, A.*, et al.* (1999). Heterozygous

germline mutations in the p53 homolog p63 are the cause of EEC syndrome. Cell *99*, 143-153.

Chene, P. (2001). The role of tetramerization in p53 function. Oncogene *20*, 2611-2617.

Cho, Y.J., Gorina, S., Jeffrey, P.D., and Pavletich, N.P. (1994). Crystal-Structure of a P53 Tumor-Suppressor DNA Complex - Understanding Tumorigenic Mutations. Science *265*, 346-355.

Clair, S.S., and Manfredi, J.J. (2006). The dual specificity phosphatase cdc25C is a direct target for transcriptional repression by the tumor suppressor p53. Cell Cycle *5*, 709-713.

Crighton, D., Wilkinson, S., O'Prey, J., Syed, N., Smith, P., Harrison, P.R., Gasco, M., Garrone, O., Crook, T., and Ryan, K.M. (2006). DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. Cell *126*, 121-134.

Delavaine, L., and La Thangue, N.B. (1999). Control of E2F activity by p21Waf1/Cip1. Oncogene *18*, 5381-5392.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Nuciforo, P.G., Bensimon, A.*, et al.* (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature *444*, 638-642.

Dominguez, G., Silva, J.M., Silva, J., Garcia, J.M., Sanchez, A., Navarro, A., Gallego, I., Provencio, M., Espana, P., and Bonilla, F. (2001). Wild type p73 overexpression and high-grade malignancy in breast cancer. Breast Cancer Res Tr *66*, 183-190.

Foster, B.A., Coffey, H.A., Morin, M.J., and Rastinejad, F. (1999). Pharmacological rescue of mutant p53 conformation and function. Science *286*, 2507-2510.

Friedler, A., Hansson, L.O., Veprintsev, D.B., Freund, S.M.V., Rippin, T.M., Nikolova, P.V., Proctor, M.R., Rudiger, S., and Fersht, A.R. (2002). A peptide that binds and stabilizes p53 core domain: Chaperone strategy for rescue of oncogenic mutants. P Natl Acad Sci USA *99*, 937-942.

Giaccia, A.J., and Kastan, M.B. (1998). The complexity of p53 modulation: emerging patterns from divergent signals. Gene Dev *12*, 2973-2983.

Green, D.R., and Kroemer, G. (2009). Cytoplasmic functions of the tumour suppressor p53. Nature *458*, 1127-1130.

Grossman, S.R., Perez, M., Kung, A.L., Joseph, M., Mansur, C., Xiao, Z.X., Kumar, S., Howley, P.M., and Livingston, D.M. (1998). p300/MDM2 complexes participate in MDM2-mediated p53 degradation. Mol Cell *2*, 405-415.

Gunther, T., Schneider-Stock, R., Hackel, C., Kasper, H.U., Pross, M., Hackelsberger, A., Lippert, H., and Roessner, A. (2000). Mdm2 gene amplification in gastric cancer correlation with expression of mdm2 protein and p53 alterations. Modern Pathol *13*, 621-626.

Halazonetis, T.D., Gorgoulis, V.G., and Bartek, J. (2008). An oncogene-induced DNA damage model for cancer development. Science *319*, 1352-1355.

Hamard, P.J., Lukin, D.J., and Manfredi, J.J. (2012). p53 Basic C Terminus Regulates p53 Functions through DNA Binding Modulation of Subset of Target Genes. J Biol Chem *287*, 22397-22407.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell *144*, 646-674.

Hermeking, H., Lengauer, C., Polyak, K., He, T.C., Zhang, L., Thiagalingam, S., Kinzler, K.W., and Vogelstein, B. (1997). 14-3-3sigma is a p53-regulated inhibitor of G2/M progression. Mol Cell *1*, 3-11.

Honda, R., and Yasuda, H. (1999). Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. Embo J *18*, 22-27.

Issaeva, N., Bozko, P., Enge, M., Protopopova, M., Verhoef, L.G.G.C., Masucci, M., Pramanik, A., and Selivanova, G. (2004). Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. Nat Med *10*, 1321-1328.

Jiang, L., Kon, N., Li, T.Y., Wang, S.J., Su, T., Hibshoosh, H., Baer, R., and Gu, W. (2015). Ferroptosis as a p53-mediated activity during tumour suppression. Nature *520*, 57-+.

Jones, S.N., Roe, A.E., Donehower, L.A., and Bradley, A. (1995). Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature *378*, 206-208.

Koblish, H.K., Zhao, S.Y., Franks, C.F., Donatelli, R.R., Tominovich, R.M., LaFrance, L.V., Leonard, K.A., Gushue, J.M., Parks, D.J., Calvo, R.R*., et al.* (2006). Benzodiazepinedione inhibitors of the Hdm2 : p53 complex suppress human tumor cell proliferation in vitro and sensitize tumors to doxorubicin in vivo. Mol Cancer Ther *5*, 160-169.

Kojima, K., Ishizawa, J., and Andreeff, M. (2016). Pharmacological activation of wild-type p53 in the therapy of leukemia. Exp Hematol *44*, 791-798.

Kumar, M., Lu, Z., Takwi, A.A.L., Chen, W., Callander, N.S., Ramos, K.S., Young, K.H., and Li, Y. (2011). Negative regulation of the tumor suppressor p53 gene by microRNAs. Oncogene *30*, 843-853.

Kussie, P.H., Gorina, S., Marechal, V., Elenbaas, B., Moreau, J., Levine, A.J., and Pavletich, N.P. (1996). Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science *274*, 948-953.

Lambert, J.M.R., Gorzov, P., Veprintsev, D.B., Soderqvist, M., Segerback, D., Bergman, J., Fersht, A.R., Hainaut, P., Wiman, K.G., and Bykov, V.J.N. (2009). PRIMA-1 Reactivates Mutant p53 by Covalent Binding to the Core Domain. Cancer Cell *15*, 376-388.

Lehmann, S., Bykov, V.J.N., Ali, D., Andren, O., Cherif, H., Tidefelt, U., Uggla, B., Yachnin, J., Juliusson, G., Moshfegh, A*., et al.* (2012). Targeting p53 in Vivo: A First-in-Human Study With p53-Targeting Compound APR-246 in Refractory Hematologic Malignancies and Prostate Cancer. J Clin Oncol *30*, 3633-3639.

Liu, D.S., Duong, C.P., Haupt, S., Montgomery, K.G., House, C.M., Azar, W.J., Pearson, H.B., Fisher, O.M., Read, M., Guerra, G.R*., et al.* (2017). Inhibiting the system x((C)over-bar)/glutathione axis selectively targets cancers with mutant-p53 accumulation. Nat Commun *8*.

Liu, D.S.H., Read, M., Cullinane, C., Azar, W.J., Fennell, C.M., Montgomery, K.G., Haupt, S., Haupt, Y., Wiman, K.G., Duong, C.P*., et al.* (2015). APR-246 potently inhibits tumour growth and overcomes chemoresistance in preclinical models of oesophageal adenocarcinoma. Gut *64*, 1506-1516.

Lohrum, M.A.E., Ludwig, R.L., Kubbutat, M.H.G., Hanlon, M., and Vousden, K.H. (2003). Regulation of HDM2 activity by the ribosomal protein L11. Cancer Cell *3*, 577-587.

Madka, V., Zhang, Y.T., Li, Q., Mohammed, A., Sindhwani, P., Lightfoot, S., Wu, X.R., Kopelovich, L., and Rao, C.V. (2013). p53-stabilizing Agent CP-31398 Prevents Growth and

Invasion of Urothelial Cancer of the Bladder in Transgenic UPII-SV40T Mice. Neoplasia *15*, 966-+.

Mahmoudi, S., Henriksson, S., Corcoran, M., Mendez-Vidal, C., Wiman, K.G., and Farnebo, M. (2009). Wrap53, a Natural p53 Antisense Transcript Required for p53 Induction upon DNA Damage. Mol Cell *33*, 462-471.

Murphy, M.E. (2016). Ironing out how p53 regulates ferroptosis. P Natl Acad Sci USA *113*, 12350-12352.

Nagata, S., and Golstein, P. (1995). The Fas Death Factor. Science *267*, 1449-1456.

Nakano, K., and Vousden, K.H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell *7*, 683-694.

Oda, K., Arakawa, H., Tanaka, T., Matsuda, K., Tanikawa, C., Mori, T., Nishimori, H., Tamai, K., Tokino, T., Nakamura, Y*., et al.* (2000). p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. Cell *102*, 849-862.

Oliner, J.D., Kinzler, K.W., Meltzer, P.S., George, D.L., and Vogelstein, B. (1992). Amplification of a Gene Encoding a P53-Associated Protein in Human Sarcomas. Nature *358*, 80-83.

Olivier, M., Eeles, R., Hollstein, M., Khan, M.A., Harris, C.C., and Hainaut, P. (2002). The IARC TP53 database: New Online mutation analysis and recommendations to users. Hum Mutat *19*, 607-614.

Olivier, M., Hollstein, M., and Hainaut, P. (2010). TP53 Mutations in Human Cancers: Origins, Consequences, and Clinical Use. Csh Perspect Biol *2*.

Ozaki, T., Naka, M., Takada, N., Tada, M., Sakiyama, S., and Nakagawara, A. (1999). Deletion of the COHH-terminal region of p73 alpha enhances both its transactivation function and DNA-binding activity but inhibits induction of apoptosis in mammalian cells. Cancer Res *59*, 5902-5907.

Parant, J., Chavez-Reyes, A., Little, N.A., Yan, W., Reinke, V., Jochemsen, A.G., and Lozano, G. (2001). Rescue of embryonic lethality in Mdm4-null mice by loss of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53. Nat Genet *29*, 92-95.

Peng, X., Zhang, M.Q.Z., Conserva, F., Hosny, G., Selivanova, G., Bykov, V.J.N., Arner, E.S.J., and Wiman, K.G. (2013). APR-246/PRIMA-1(MET) inhibits thioredoxin reductase 1 and converts the enzyme to a dedicated NADPH oxidase. Cell Death Dis *4*.

Puzio-Kuter, A.M. (2011). The Role of p53 in Metabolic Regulation. Genes Cancer *2*, 385-391.

Rodriguez, M.S., Desterro, J.M.P., Lain, S., Lane, D.P., and Hay, R.T. (2000). Multiple C-terminal lysine residues target p53 for ubiquitin-proteasome-mediated degradation. Mol Cell Biol *20*, 8458-8467.

Romano, R.A., Smalley, K., Magraw, C., Serna, V.A., Kurita, T., Raghavan, S., and Sinha, S. (2012). Delta Np63 knockout mice reveal its indispensable role as a master regulator of epithelial development and differentiation. Development *139*, 772-782.

Sakaguchi, K., Saito, S., Higashimoto, Y., Roy, S., Anderson, C.W., and Appella, E. (2000). Damage-mediated phosphorylation of human p53 threonine 18 through a cascade mediated by a casein 1-like kinase - Effect on Mdm2 binding. J Biol Chem *275*, 9278-9283.

Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J., and Howley, P.M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell *63*, 1129-1136.

Scherer, S.J., Welter, C., Zang, K.D., and Dooley, S. (1996). Specific in vitro binding of p53 to the promoter region of the human mismatch repair gene hMSH2. Biochem Bioph Res Co *221*, 722-728.

Schon, O., Friedler, A., Bycroft, M., Freund, S.M.V., and Fersht, A.R. (2002). Molecular mechanism of the interaction between MDM2 and p53. J Mol Biol *323*, 491-501.

Scotcher, J., Clarke, D.J., Weidt, S.K., Mackay, C.L., Hupp, T.R., Sadler, P.J., and Langridge-Smith, P.R.R. (2011). Identification of Two Reactive Cysteine Residues in the Tumor Suppressor Protein p53 Using Top-Down FTICR Mass Spectrometry. J Am Soc Mass Spectr *22*, 888-897.

Shaulsky, G., Goldfinger, N., Benzeev, A., and Rotter, V. (1990). Nuclear Accumulation of P53 Protein Is Mediated by Several Nuclear-Localization Signals and Plays a Role in Tumorigenesis. Mol Cell Biol *10*, 6565-6577.

Shimada, A., Kato, S., Enjo, K., Osada, M., Ikawa, Y., Kohno, K., Obinata, M., Kanamaru, R., Ikawa, S., and Ishioka, C. (1999). The transcriptional activities of p53 and its homologue p51/p63: Similarities and differences. Cancer Res *59*, 2781-2786.

Skulachev, V.P. (1998). Cytochrome c in the apoptotic and antioxidant cascades. Febs Lett *423*, 275-280.

Sommer, M., Poliak, N., Upadhyay, S., Ratovitski, E., Nelkin, B.D., Donehower, L.A., and Sidransky, D. (2006). Delta Np63 alpha overexpression induces downregulation of Sirt1 and an accelerated aging phenotype in the mouse. Cell Cycle *5*, 2005-2011.

Spinnler, C., Hedstrom, E., Li, H., de Lange, J., Nikulenkov, F., Teunisse, A.F.A.S., Verlaan-de Vries, M., Grinkevich, V., Jochemsen, A.G., and Selivanova, G. (2011). Abrogation of Wip1 expression by RITA-activated p53 potentiates apoptosis induction via activation of ATM and inhibition of HdmX. Cell Death Differ *18*, 1736-1745.

Stiewe, T., and Putzer, B.M. (2002). Role of p73 in malignancy: tumor suppressor or oncogene? Cell Death Differ *9*, 237-245.

Stommel, J.M., Marchenko, N.D., Jimenez, G.S., Moll, U.M., Hope, T.J., and Wahl, G.M. (1999). A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. Embo J *18*, 1660-1672.

Suzuki, D., Sahu, R., Leu, N.A., and Senoo, M. (2015). The carboxy-terminus of p63 links cell cycle control and the proliferative potential of epidermal progenitor cells. Development *142*, 282-290.

Talos, F., Abraham, A., Vaseva, A.V., Holembowski, L., Tsirka, S.E., Scheel, A., Bode, D., Dobbelstein, M., Bruck, W., and Moll, U.M. (2010). p73 is an essential regulator of neural stem cell maintenance in embryonal and adult CNS neurogenesis. Cell Death Differ *17*, 1816-1829.

Tannapfel, A., Wasner, M., Krause, K., Geissler, F., Katalinic, A., Hauss, J., Mossner, J., Engeland, K., and Wittekind, C. (1999). Expression of p73 and Its relation to histopathology and prognosis in hepatocellular carcinoma. J Natl Cancer I *91*, 1154-1158.

Tasdemir, E., Maiuri, M.C., Morselli, E., Criollo, A., D'Amelio, M., Djavaheri-Mergny, M., Cecconi, F., Tavernarakis, N., and Kroemer, G. (2008). A dual role of p53 in the control of autophagy. Autophagy *4*, 810-814.

Tessoulin, B., Descamps, G., Moreau, P., Maiga, S., Lode, L., Godon, C., Marionneau-Lambot, S., Oullier, T., Le Gouill, S., Amiot, M.*, et al.* (2014). PRIMA-1(Met) induces myeloma cell death independent of p53 by impairing the GSH/ROS balance. Blood *124*, 1626-1636.

Thomas, M., Pim, D., and Banks, L. (1999). The role of the E6-p53 interaction in the molecular pathogenesis of HPV. Oncogene *18*, 7690-7700.

Varley, J.M. (2003). Germline TP53 mutations and Li-Fraumeni syndrome. Hum Mutat *21*, 313-320.

Vassilev, L.T., Vu, B.T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C.*, et al.* (2004). In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science *303*, 844-848.

Victorelli, S., and Passos, J.F. (2017). Telomeres and Cell Senescence - Size Matters Not. EBioMedicine *21*, 14-20.

Vilborg, A., Glahder, J.A., Wilhelm, M.T., Bersani, C., Corcoran, M., Mahmoudi, S., Rosenstierne, M., Grander, D., Farnebo, M., Norrild, B.*, et al.* (2009). The p53 target Wig-1 regulates p53 mRNA stability through an AU-rich element. P Natl Acad Sci USA *106*, 15756-15761.

Wassman, C.D., Baronio, R., Demir, O., Wallentine, B.D., Chen, C.K., Hall, L.V., Salehi, F., Lin, D.W., Chung, B.P., Hatfield, G.W.*, et al.* (2013). Computational identification of a transiently open L1/S3 pocket for reactivation of mutant p53. Nat Commun *4*.

Weyemi, U., Lagente-Chevallier, O., Boufraqech, M., Prenois, F., Courtin, F., Caillou, B., Talbot, M., Dardalhon, M., Al Ghuzlan, A., Bidart, J.M.*, et al.* (2012). ROS-generating NADPH oxidase NOX4 is a critical mediator in oncogenic H-Ras-induced DNA damage and subsequent senescence. Oncogene *31*, 1117-1129.

Wischhusen, J., Naumann, U., Ohgaki, H., Rastinejad, F., and Weller, M. (2003). CP-31398, a novel p53-stabilizing agent, induces p53-dependent and p53-independent glioma cell death. Oncogene *22*, 8233-8245.

Yang, A., Walker, N., Bronson, R., Kaghad, M., Oosterwegel, M., Bonnin, J., Vagner, C., Bonnet, H., Dikkes, P., Sharpe, A.*, et al.* (2000). p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. Nature *404*, 99-103.

Yu, X., Blanden, A.R., Narayanan, S., Jayakumar, L., Lubin, D., Augeri, D., Kimball, S.D., Loh, S.N., and Carpizo, D.R. (2014). Small molecule restoration of wildtype structure and function of mutant p53 using a novel zinc-metallochaperone based mechanism. Oncotarget *5*, 8879-8892.

Yu, X., Vazquez, A., Levine, A.J., and Carpizo, D.R. (2012). Allele-Specific p53 Mutant Reactivation. Cancer Cell *21*, 614-625.

Zache, N., Lambert, J.M.R., Rokaeus, N., Shen, J.F., Hainaut, P., Bergman, J., Wiman, K.G., and Bykou, V.N. (2008). Mutant p53 targeting by the low molecular weight compound STIMA-1. Mol Oncol *2*, 70-80.

Zandi, R., Selivanova, G., Christensen, C.L., Gerds, T.A., Willumsen, B.M., and Poulsen, H.S. (2011). PRIMA-1(Met)/APR-246 Induces Apoptosis and Tumor Growth Delay in Small Cell Lung Cancer Expressing Mutant p53. Clin Cancer Res *17*, 2830-2841.

Zhu, J.H., Jiang, J.Y., Zhou, W.J., and Chen, X.B. (1998). The potential tumor suppressor p73 differentially regulates cellular p53 target genes. Cancer Res *58*, 5061-5065.

Zou, N., Xie, G.Z., Cui, T.T., Srivastava, A.K., Qu, M.H., Yang, L.L., Wei, S.Z., Zheng, Y.F., and Wang, Q.E. (2016). DDB2 increases radioresistance of NSCLC cells by enhancing DNA damage responses. Tumor Biol *37*, 14183-14191.

Zou, T.T., Mazan-Mamczarz, K., Rao, J.N., Liu, L., Marasa, B.S., Zhang, A.H., Xiao, L., Pullmann, R., Gorospe, M., and Wang, J.Y. (2006). Polyamine depletion increases cytoplasmic levels of RNA-binding protein HuR leading to stabilization of nucleophosmin and p53 mRNAs. J Biol Chem *281*, 19387-19394.